THE PURIFICATION OF HEPARIN AND ITS CHEMICAL AND PHYSIOLOGICAL REACTIONS

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THE PURIFICATION OF HEPARIN AND ITS CHEMICAL AND PHYSIOLOGICAL REACTIONS[1]

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An anticoagulant obtained from the dog's liver was described by Howell and Holt in 1918 (1). This substance was prepared from an ethereal extract of liver dried at a temperature of 60° to 80°F., and was designated provisionally as heparin. Owing to the method of preparation and its content in phosphorus it was assumed, erroneously, to belong to the group of phosphatids. The anticoagulant action of this material, although distinct, was not very strong, 1 milligram being required to prevent the coagulation of 1 to 2 cc. of blood. Since the product obtained proved to be soluble in water and insoluble in alcohol and ether, Howell in 1922 (2) devised a different method of preparation, starting with an aqueous or saline extract of the dried liver. The material obtained by this method exhibited a much stronger anticoagulant action, 1 milligram being sufficient to prevent clotting of 5 cc. of blood. Intravenous injections of its solution caused no toxic reactions and when used in proper concentrations made the blood of the animal entirely incoagulable for a certain period. Since the potency of this preparation was of the same order as that of hirudin, and since the latter material was difficult to procure, the firm of Hynson, Westcott and Dunning, of Baltimore, undertook to prepare heparin commercially for the use of laboratory workers desiring a suitable anticoagulant for experimental purposes. The method used in its preparation did not yield a constant product, although the material could be standardized to a definite potency. It was evident, however, that the active element was mixed with more or less inactive material, and repeated efforts have been made to isolate the substance in at least an approximate state of purity in order to determine its probable chemical nature. Numerous procedures used for

[1] Received for publication January 19, 1928.

this purpose (3) were successful in increasing the potency of the preparation. Products were obtained in which 1 mgm. sufficed to prevent clotting of 20 or even 40 cc. of blood and which gave no reaction for phosphorus. This last result demonstrated that the active substance is not a phosphatid as was supposed at first. Lately a method has been devised which gives the substance in apparently pure form, in a form at least which shows constant reactions and which is free from both nitrogen and phosphorus. The anticoagulant action for human blood is of the order of 1 mgm. to 100 cc. of blood.

METHOD

The method consists essentially of two procedures. First, adsorption of impurities by Lloyd's reagent in acid solution, and second, precipitation of the active substance by barium hydroxide. Repeated treatment with the Lloyd's reagent alone in 4 per cent acetic acid gives a preparation that is very active so that 1 mgm. will prevent the clotting of 50 cc. of human blood. The further purification by barium precipitation is attended by considerable loss of material, but yields a purer and more active substance. The details are as follows. Crude heparin prepared by the method of 1922 (2) was dissolved in water, 1 gram of heparin to 50 cc. of water, and digested for 6 to 12 hours at body temperature with taka-diastase to hydrolyze any glycogen that might be present. The solution was then filtered, evaporated on a water-bath nearly to dryness and boiled with a large volume of 95 per cent methyl alcohol for an hour, a reflux condenser being employed. The alcohol was removed by filtration and evaporation, the solid residue was dissolved in 1 per cent solution of sodium chloride, filtered and precipitated by the addition of an equal volume of acetone. This precipitate was collected by centrifugalizing, washed with 50 per cent acetone, dissolved in water, and treated with Lloyd's reagent in acid solution. Three successive treatments were used with this reagent in 2, 4, and 8 per cent acetic acid. After each treatment the solution was filtered first through a Buchner filter and then through filter paper. The final solution was neutralized with dilute sodium hydroxide and precipitated by the addition of an equal volume of acetone. The precipitate was collected by centrifugalizing, washed, dried, dissolved in water and filtered. This solution was then treated

with an excess of a saturated solution of barium hydroxide. The white precipitate was centrifugalized off, washed with water by layering in the centrifugal tube, and then stirred up with water and dilute hydrochloric acid added to distinct acid reaction. This mixture was centrifugalized and the clear supernatant liquid was poured off. The barium in this solution was precipitated by very careful addition of sodium sulphate or sulphuric acid, avoiding an excess. The barium precipitate was centrifugalized off, the supernatant liquid was neutralized with dilute sodium hydroxide and then after filtration evaporated to dryness on a water-bath. The residue obtained was treated with water. If it did not all dissolve the solution was filtered, evaporated to dryness and dissolved in water. This treatment was repeated until the residue obtained on evaporation dissolved completely in water. The final solution was precipitated by the addition of an equal volume of acetone. To ensure the separation out of a precipitate, sodium acetate was added to a concentration of one half per cent. The precipitate was centrifugalized off, washed with 50 per cent acetone and finally with acetone and ether. The material thus obtained is amorphous and readily soluble in water, although insoluble in all other solvents used. When its aqueous solutions are evaporated over the water-bath it is deposited in the form of a transparent glassy membrane. It was found that solutions of crude heparin and of the product obtained after treatment with the Lloyd's reagent give a blue color with the phenol reagent of Folin and Dennis, but after purification by barium precipitation the final product should give no trace of this reaction.

REACTIONS

Aqueous solutions of the purified heparin give the following reactions.

1. A negative test for phosphorus and nitrogen (sodium cyanide test).
2. It is not precipitated by salts of copper, zinc, cadmium, nickel, iron or platinum. It is precipitated by basic acetate of lead.
3. Barium hydroxide gives a white precipitate which turns canary yellow on boiling.
4. It gives the alpha naphthol reaction for carbohydrates.
5. Treated with Fehling's solution it gives no reduction, but if

first hydrolyzed thoroughly with dilute acid (HCl), it reduces Fehling's solution readily.

6. It gives no color reaction with iodine.

7. It gives a positive reaction for glycuronic acid with naphthoresorcin. The test was made according to the directions given by Tollens. To the heparin solution was added an alcoholic solution of naphthoresorcin, 0.1 cc. for each cubic centimeter of solution, and then an equal volume of concentrated HCl. The mixture was boiled gently for a minute and then allowed to cool for 3 or 4 minutes. Addition of ether gave a red violet layer showing an absorption band at D. When benzol was used in place of ether, the coloration took place much more slowly. It should be stated, however, that when the "Absatz" method was used the alcoholic solution instead of showing a violet color was reddish with a green fluorescence by reflected light.

8. It gives a positive reaction for glycuronic acid with orcinol (Bial's reagent). Two cubic centimeters of the Bial's reagent (HCl 100 cc., orcinol 200 mgm., ferric chloride (liq. ferri sesquichl.) 4 drops) were boiled and the heparin solution added a drop at a time. A fine green color developed, as is the case with glycuronic acid and the pentoses. In aqueous solution or after extraction with amyl alcohol an absorption band was obtained between D and C. When orcinol was used without the iron (Salkowski's method) this color reaction was not obtained, but the same negative result followed when glycuronic acid, prepared from euxanthinic acid, was tested in the same way. Although this reaction with Bial's reagent is given by both glycuronic acid and pentoses, the result of the test with naphthoresorcin excludes the latter group.

9. Reaction with parabromphenylhydrazine. This reaction was negative. Neuberg (4) has described a crystalline hydrazine compound of glycuronic acid and p bromphenylhydrazine which is characterized by its insolubility in alcohol and its high specific rotation when dissolved in a mixture of pyridine and absolute alcohol. It has been claimed that this reaction constitutes the most positive test for glycuronic acid. Goldschmiedt and Zerner (5), on the contrary, state that the reaction is dependent upon conditions that are uncontrollable and that it is not a satisfactory test for glycuronic acid. My experience agrees with this conclusion.

10. Hydrolysis with dilute mineral acids destroys its anticoagulant action.

11. When boiled with dilute hydrochloric acid (1–4) and evaporated to dryness a white residue is obtained which is soluble in water but not in alcohol. Aqueous solutions of the residue reduce Fehling's solutions and give the reactions of glycuronic acid. The same treatment with nitric acid (s.g., 1.15) gives after evaporation a sticky residue, part or all of which is soluble in alcohol, together with a granular or crystalline inorganic deposit. Aqueous solutions of the residue reduce Fehling. Tests of these solutions with barium chloride and ammonium oxalate indicate the presence of sulphuric acid and calcium. If during the purification of the heparin, after treatment with Lloyd's reagent but before precipitation with barium hydroxide, some of the material is treated upon the water-bath with nitric acid (s. g. 1.15) and the solution is evaporated to a small bulk, as in the process of the production of mucic acid from galactose, crystals of calcium sulphate separate out.

12. Elementary analyses of the heparin for carbon, hydrogen and ash give the following figures O, 20.5 per cent; H, 3.75 per cent; Ash, 37 per cent.[2]

The conclusion drawn from these reactions is that the heparin is a glycuronic acid compound containing, as isolated, probably calcium and sulphuric acid in the molecule. The fact that it shows reducing properties only after acid hydrolysis would indicate that it is either a paired glycuronate or a condensation product of glycuronic acid. The low carbon percentage would favor the latter interpretation.

PHYSIOLOGICAL REACTIONS

The purified heparin shows the two characteristic reactions that have been described in previous papers, namely, an anticoagulant action upon blood and the property of causing an increase in antithrombin when added to plasma or serum. In concentrations of about 1 mgm. to 100 cc. human blood is prevented from clotting for 24 hours or longer. The blood seems to be preserved in an entirely normal

[2] For this analysis I am indebted to the kindness of Dr. Wintersteiner of the department of Pharmacology of the Johns Hopkins Medical School.

condition. The corpuscles settle rapidly as is the case with other anticoagulants and with the slowly clotting blood of hemophilia. Even after 24 hours the platelets may be found single, that is, there is little or no agglutination. If the platelets are pipetted off from the supernatant plasma and treated with water they quickly agglutinate and dissolve. For this reason the heparinized blood, when diluted freely with water, soon shows clotting. If, however, a fresh specimen of heparinized blood is centrifugalized at high speed for an hour and the upper layers are then removed and diluted with water, spontaneous clotting does not occur. The anticoagulant action of heparin is not destroyed by prolonged boiling of its solutions. Its aqueous solutions are also apparently highly resistant to the action of bacteria. A very dilute solution that was left over summer, a period of three and a half months, in a corked vial remained perfectly clear and exhibited the same anticoagulant action as when freshly prepared.

In regard to its antithrombic action it was found that heparin itself is not an antithrombin, that is, it has no effect upon the coagulating properties of active thrombin, nevertheless, when added to plasma or serum, it causes a marked increase in the amount of antithrombin present in these liquids, as determined by the effect of the mixture upon the reaction between thrombin and fibrinogen.

When the heparin is injected into the circulation of a normal animal in proper concentrations the blood is rendered wholly incoagulable for a certain period. In the case of a dog an injection of 1 mgm. to each 50 cc. of the estimated amount of blood (1/20) keeps the blood incoagulable for a period of about half an hour. In an hour the coagulability begins to return. Specimens withdrawn after this time may show imperfect clotting in 3 to 4 hours and complete coagulation later on (6 to 24 hours). After two hours specimens withdrawn may clot in 30 to 40 minutes and after three hours the coagulation of the blood is practically back to normal. During the first hour or two after injection the animal exhibits hemophilic oozing from the skin wounds made in the exposure of the blood vessels.

THE USE OF HEPARIN IN TRANSFUSIONS

The strong anticoagulant action of heparin, the absence of injurious reactions when it is injected intravenously, and the fact that its solu-

tions are very stable and can be readily sterilized by boiling suggested that the purified product might be useful in the transfusion of blood in human beings. Through the kindness of Dr. Longcope its applicability for such purposes was tested upon several patients in the medical wards of the Johns Hopkins Hospital. A solution was made of the purified heparin in 0.9 per cent solution of sodium chloride of such a strength that 1 milligram was contained in each 10 cc. of solution. The saline solution was made from freshly distilled water to avoid the products of bacterial growth that are known to be formed in distilled water that has been kept for some time. The solution of heparin was sterilized by boiling and enough was made at one time for a series of transfusions. In collecting the blood to be transfused it was allowed to run directly from the vein of the donor into a sterile flask containing the heparin solution, 10 cc. of the latter being used for each 100 cc. of blood to be transfused.

Ten transfusions were made upon six patients, one with primary anemia, the others with severe secondary anemias. In eight of the transfusions no reaction was observed, the amounts of blood transfused varying from 300 to 500 cc. In the other two cases there was a slight chill at one and a quarter and two hours after the transfusion, lasting for twenty-five to thirty minutes with a temperature of 102°F.

THE ANTICOAGULANT ACTION OF GLYCURONIC ACID

Since the heparin is probably a derivative of glycuronic acid it seemed desirable to prepare glycuronic acid and test its effect upon coagulation. For this purpose I was fortunate enough to obtain some Purree, the raw material from which Indian Yellow was formerly made, through the courtesy of Messrs. Winsor and Newton Ltd. of London. The Purree contains glycuronic acid as a magnesia compound of euxanthinic acid. The glycuronic acid was obtained from Purree by a modification of the process described by Thierfelder (6). The euxanthinic acid was obtained from the magnesia salt by the action of hydrochloric acid and was recrystallized from hot alcohol solutions. This acid was then hydrolyzed by one per cent sulphuric acid in an autoclave at a temperature of 125°C. The insoluble euxanthon was filtered off and the solution of glycuronic acid, after neutralization, was evaporated to one-third its volume before an

electric fan, filtered, and then carried to dryness on the water-bath. The residue was extracted repeatedly with a mixture consisting of alcohol 1 volume, ether 1 volume, and water 0.4 volume. From this extract the glycuronic acid was obtained by evaporation. The material obtained reduces Fehling's solution and gives the naphthoresorcin and orcinol reactions. The glycuronic acid as thus obtained has no anticoagulant action, but it was found that when heated to 250°C. it forms a dark-colored compound easily soluble in water, which has a very definite inhibitory effect upon coagulation. One milligram will prevent the coagulation of 1 to 2 cc. of human blood for 24 hours or longer. This compound and other modifications of glycuronic acid are now being studied from this point of view.

SUMMARY

1. A method is described for the purification of crude heparin which gives a product free from phosphorus and nitrogen. Its anticoagulant action for human blood is of the order of 1 mgm. to 100 cc. of blood.

2. The reactions of the purified heparin show that it is a carbohydrate body, apparently a glycuronic acid compound.

3. The use of the purified heparin as a safe anticoagulant for blood transfusions is suggested.

4. Glycuronic acid prepared from euxanthinic acid has no anticoagulant action, but on heating to 250°C. it yields a compound with distinct anticoagulant properties.

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